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(54) **METHOD FOR INHIBITING DECOMPOSITION OF NATRIURETIC PEPTIDES AND IMPROVED METHOD FOR ASSAYING NATRIURETIC PEPTIDES WITH THE USE OF THE SAME**

(57) A method for inhibiting the degradation of mammalian natriuretic peptides, in particular, BNP by using containers wherein the face coming into contact with specimens are made of a material capable of inhibiting the

activation of a substance degrading peptides. This method makes it possible to collect specimens for measuring natriuretic peptides stably and conveniently.

Also provided is a method for measuring natriuretic peptides by using these containers.

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## Description

## Technical Field

5 This invention relates to methods for inhibiting the degradation of natriuretic peptides by using a container which comprises a material inhibiting the activation of a substance degrading the peptides and also relates to methods for measuring, assaying, collecting, and storing of the peptides by using the container.

## Background Art

10 A natriuretic peptide family consists of at least three types of natriuretic peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C type natriuretic peptide (CNP). CNP is a vascular proliferation-regulating peptide mainly secreted from endothelial cells. ANP and BNP are cardiac hormones mainly synthesized in and secreted from heart. These peptides are synthesized as pro-hormones and cleaved to be mature peptides,  $\alpha$ -ANP,  $\alpha$ -BNP,  $\alpha$ -CNP respectively.

15 Human  $\alpha$ -ANP,  $\alpha$ -BNP, and  $\alpha$ -CNP consist of 28, 32, and 22 amino acid residues, respectively. Some diseases cause the secretion of these natriuretic peptides into blood stream. Since the synthesis and secretion of ANP and BNP are promoted mainly by a load against atria and ventricles of heart, respectively, their secretions reflect changes of heart functions. Each peptide is used as a diagnostic indicator of heart diseases, especially heart failure. Measurement of both  $\alpha$ -ANP and  $\alpha$ -BNP by immunoassay has already been applied in the clinical field.

20 Since  $\alpha$ -ANP and  $\alpha$ -BNP are easily degraded by proteases in blood after the collection, they are extremely unstable in blood samples. Thus results of measurement had been greatly affected by the collecting methods, storing methods of specimens and the period from collection to measurement. To measure the concentration of the peptides exactly, addition of degradation inhibiting agents, e.g., aprotinin etc. or keeping specimens at low temperature had been essential. But, these handlings were complicate, required too many tasks, and not completed methods as pretreatment of specimens.

## Disclosure of Invention

25 It is speculated that after blood collection natriuretic peptides are degraded by substances such as proteases in blood. To date, some protease inhibitors were added into the samples for the inhibition of the degradation of natriuretic peptides. But, it could not completely inhibit the degradation. The present inventors have speculated that coagulation factors activated by negatively charged solid phase such as glass surface accelerate the degradation of natriuretic peptides when specimens are collected into a container made of glass. The inventors have collected specimens by using a glass container wherein the face coming into contact with a specimens was coated with silicon, and obtained a result that the degradation of natriuretic peptides were inhibited.

30 The inventors have found out that the degradation of natriuretic peptides by a substance such as proteases can be suppressed significantly by using a container coated with silicone upon measurement of natriuretic peptide.

The inventors have also found out that the degradation of natriuretic peptides can be suppressed by using a container made of plastic such as polyethylene terephthalate (PET), polystyrene, polypropylene, polyethylene and acrylic resin.

35 These results suggest that the degradation of natriuretic peptides in specimens can be suppressed by using a container wherein the face coming into contact with specimens is made of a material inhibiting the activation of a substance degrading the peptides upon handling specimens containing mammalian natriuretic peptides. Therefore, it is expected that the former complicated handling of specimens can be eliminated by using a container wherein the face coming into contact with specimens is made of materials other than glass upon the measurement of natriuretic peptides. Further expected is that these convenient specimens collecting methods for sample preparation give more exact results for diagnosis of heart diseases than conventional methods already used in the clinical field.

40 This invention is based on the results of the measurement of natriuretic peptides by thus established methods for the inhibition of degradation of mammalian natriuretic peptides by using a container which do not activate substances degrading the peptides in handling specimens containing the peptides.

45 This invention relates to a method for inhibiting the degradation of mammalian natriuretic peptides by using a container wherein the face coming into contact with specimens made of a materials, preferably, silicone or plastic, which inhibits the activation of the substances degrading the peptides.

50 Mammalian natriuretic peptides comprise at least ANP and BNP and precursors and derivatives of each peptide because in body there are not only the mature types but also the precursors such as  $\gamma$ -ANP and  $\gamma$ -BNP (BBRC, 214(3), (1995)), and their derivatives. Mammal means all kinds of mammal having natriuretic peptides, such as human, dog, pig, rat and mouse.

55 "Handling of specimens" means any kinds of handling for specimens, such as collection, storage, analysis, measurement and so on of the specimens.

"Materials inhibiting the activation of a substance degrading peptides" mean materials, which can inhibit the activation of

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substances degrading the peptides, such as proteases etc., and can at least form the face coming into contact with the specimen contained in a specimen collecting container. Examples of the material include silicone and plastic, preferably polyethylene, polyethylene terephthalate, polystyrene, polypropylene, polyamide, acrylic resin and so on. SILICONIZE L-25 (Ficon Co.) is given for example as commercially available silicone. It is possible for persons skilled in the art to coat usually used containers made of glass and polyethylene with silicone by using this reagent.

"Container" means all kinds of containers for specimen collection, storage, measurement and so on, for example, a container which is made of or coated with a material inhibiting the degradation, preferably, with silicone or plastic.

Any kind of biological samples can be used for measuring specimens, and preferred is whole blood or blood plasma.

This invention relates to a measurement of natriuretic peptide in specimens which do not contain aprotinin.

Although aprotinin has been added into specimens to inhibit the degradation of natriuretic peptides by proteases which are already active in blood or are activated after blood collection, it can not inactivate them contained in biological samples completely.

This invention relates to a measuring method of mammalian natriuretic peptides which comprises the method for inhibiting the degradation of the peptides.

The measurement of natriuretic peptides can be carried out by a biological activity measurement, liquid chromatography, immunoassay and so on. The immunoassay can be performed, which may be competitive immunoassay or sandwich immunoassay, by persons skilled in the art. Otherwise, commercially available  $\alpha$ -ANP assay kit "SHIONORIA ANP" (Shionogi & Co., Ltd.) or  $\alpha$ -BNP assay kit "SHIONORIA BNP" (Shionogi & Co., Ltd.) can also be used for the measurement.

Furthermore, this invention relates to a kit for measuring mammalian natriuretic peptides. The kit comprises the method for inhibiting the degradation of the peptides in a specimen by using a container wherein the face coming into contact with the specimen is made of a material inhibiting the activation of a substance degrading the peptides upon the specimen collection or measurement.

### Brief Description of Drawings

Fig. 1 shows the relationship between the storing periods in glass tubes or silicone-coated glass tubes at 25°C and the residual activities of BNP like substances measured by various kinds of BNP measuring methods.

Fig. 2 shows the relationship between the storing periods in silicone-coated or non-coated polyethylene terephthalate tubes or glass tubes at 25 °C and the residual activities of BNP like substances.

Fig. 3 shows the residual BNP activities of BNP like substances stored in silicone-coated or non-coated glass tube and various kinds of plastic tubes, such as polystyrene, polypropylene, reinforced polyethylene and acrylic resin for 24 hours at 25°C.

### Example

More detail of this invention is explained in the following examples, which does not limit this invention.

#### Example 1

##### Measurement of BNP using glass tubes

(1) Preparation of silicone coated glass tubes: Commercially available glass tubes (Terumo, Tokyo, Japan) were washed with purified water once, and with 3 % (V/V) silicone solution (SILICONIZE L-25: Ficon Co.) three times. They were washed once again with purified water and dried for 90 min at 300 °C

(2) Preparation of a specimen for measurement: Venous blood from normal subject was collected into a blood-collecting tube containing EDTA (1.5 mg/ml EDTA-2Na). Human  $\alpha$ -BNP (Peptide Institute, Osaka, Japan) was added to the collected blood to make its final concentration 200 pg/ml, to prepare a specimen.

(3) BNP measurement by IRMA method: The specimen was pipetted into the silicone-coated tubes and the non-coated tubes, respectively. They were allowed to stand for 0, 2, 6, and 24 hours at room temperature (25 °C). Blood

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cells were separated from these specimens by a centrifugation (Kokusan: H-107GA),  $\times 2000$  g, for 5 min at 4°C. These specimens were stored at - 80 °C. BNP immunoreactivities were measured by SHIONORIA BNP (Shionogi).

Briefly, 100 $\mu$ l of plasma or standard solution ( $\alpha$ -BNP solutions: 0, 4, 10, 150, 600, and 2000 pg/ml), were pipetted into Shionogi tubes (made of polystyrene: Shionogi), respectively. Two hundreds  $\mu$ l of iodine labeled anti-BNP antibody solution and a anti-BNP antibody immobilized polystyrene bead were added into the tubes. The mixture was stirred and then left alone for 18 hours at 4°C. After washing twice with 2 ml of washing solution, the radioactivities were measured by  $\gamma$ -counter ARC-600 (Aloka). The result is shown in Fig.1.

In the case of using non-coated glass tubes (Figure 1,  $\S$ ), the ratio of residual BNP activity was about 20% after 24 hours-standing. On the other hand, the residual BNP activity ratio was about 80% even after 24 hours-standing and the activity of substances degrading peptides was suppressed by using the silicone-coated glass tubes (Fig.1,  $\%$ ).

Fig.1 shows that the activity of substances degrading natriuretic peptides can be suppressed by silicone-coating the face coming into contact with the specimen in a specimen collecting container.

### Example 2

Measurement of BNP using polyethylene terephthalate(PET) tubes

(1) Preparation of silicon-coated PET tubes: Commercially available PET tubes (Terumo, Tokyo, Japan) were washed with purified water once, and with 3 % (V/V) silicone solution (SILICONIZE L-25: Ficon Co.) three times. They were washed once again with purified water and dried.

(2) Preparation of a specimen for measurement: Fifty ml of venous blood from normal subject was collected into a blood-collecting tubes containing EDTA (1.5 mg/ml EDTA-2Na). Human  $\alpha$ -BNP (Peptide Institute) was added to the collected blood to make its final concentration 200 pg/ml, to prepare a specimen.

(3) BNP measurement by IRMA method: The specimen was pipetted into the silicone-coated PET tubes, the silicone-coated glass tubes, the non-coated PET tubes and the non-coated glass tubes, respectively. They were allowed to stand for 0, 2, 6, 24, and 72 hours at room temperature (25 °C). Blood cells were separated from these specimens by a centrifugation (Kokusan: H-107GA),  $\times 2000$  g, for 5 min at 4°C. These specimens were stored at - 80 °C. BNP immunoreactivities in these blood plasma were measured by SHIONORIA BNP (Shionogi). The measurement was performed by the same method as that described in Example 1.

The result is shown in Fig.2. The ratio of residual BNP activity was about 80% after 24 hours-standing due to the suppression of the activity of substances degrading peptides by using the silicone-coated PET tubes (Fig.2,  $\alpha$ ) and the non-coated PET tubes (Fig.2,  $\bullet$ ). The result was the same as that of using the silicone-coated glass tubes (Fig.2,  $\%$ ). On the other hand, the ratio of residual BNP activity was 0 % after 24 hours-standing by using the non silicone-coated glass tubes(Fig.2,  $\S$ ).

### Example 3

Measurement of BNP using plastic tubes

As specimen storing containers, glass tubes, silicon coated glass tubes, and plastic tubes were used. Five kinds of plastic tubes, i.e., polystyrene tubes, polypropylene A tubes, polypropylene B tubes, reinforced polyethylene tubes, and acrylic resin tubes were used.

#### (1) BNP measurement by IRMA method

The specimen was pipetteed into each of the above described plastic tubes, coated with or without silicone. They were allowed to stand for 0, and 24 hours at room temperature (25 °C). Blood cells were separated from these specimens by a centrifugation (Kokusan: H-107GA),  $\times 2000$  g, for 5 min at 4°C. The obtained plasma specimens were stored at - 80°C. BNP immunoreactivities in these plasma specimens were measured by SHIONORIA BNP (Shionogi). The measurement was performed by the same method as Example 1.

The ratios of the residual BNP activities were 50% or more due to the suppression of the activity of substances

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degrading peptides by using any kinds of plastic tube used, i.e., polystyrene tube, polypropylene A tube, polypropylene B tube, reinforced polyethylene tube, and acrylic resin tube (Figure 3, lane 3, 4, 5, 6, and 7). The result was the same as that by using the silicone-coated glass tube (Figure 3, lane 2). On the other hand, the ratio of residual BNP activity was 0 % by using the non-coated glass tube (Fig.3, lane 1).

The residual activity of BNP remarkably decreased in glass tubes because BNP was degraded by substances degrading the peptides such as proteases. While the decrease of the residual BNP activity was suppressed in silicon-coated glass tubes. Furthermore, in plastic tubes made of polyethylene terephthalate, polystyrene, polypropylene, polyethylene or acrylic resin coated with or without silicone, the degradation of BNP was suppressed due to the inhibition of the activation of substances degrading peptides.

## Effect of Invention

The method of this invention for inhibiting the peptide degradation by using a container wherein the face coming into contact with a specimen is made of a materials inhibiting the activation of the degrading substances, provides stable and dependable clinical data on which collecting methods, storing methods and period till measurement do not have any effects.

Further, it will contribute to an exact diagnosis of heart disease by providing economical, convenient stable and dependable clinical data because blood samples can be used for measuring without complicate handling.

## Claims

1. A method for inhibiting the degradation of mammalian natriuretic peptides in specimen, which comprises using, upon handling the specimen, a container wherein the face coming into contact with the specimen is made of or coated with a material inhibiting the activation of a substance degrading the peptides.
2. The method as claimed in claim 1, wherein said material is silicone or plastic.
3. The method as claimed in claim 1 or 2, wherein said mammal is human, dog, pig, rat and mouse.
4. The method as claimed in any one of claims 1 to 3, wherein said natriuretic peptide is BNP.
5. The method as claimed in any one of claims 1 to 4, wherein said specimen does not contain aprotinin.
6. A method for measuring mammalian natriuretic peptides, which comprises the method as claimed in claim 1.
7. A kit for measuring mammalian natriuretic peptides, which comprises a container wherein the face coming into contact with specimen is made of or coated with a material inhibiting the activation of a substance degrading the peptides.
8. The kit as claimed in claim 7, wherein said specimen does not contain aprotinin.

Figure 1

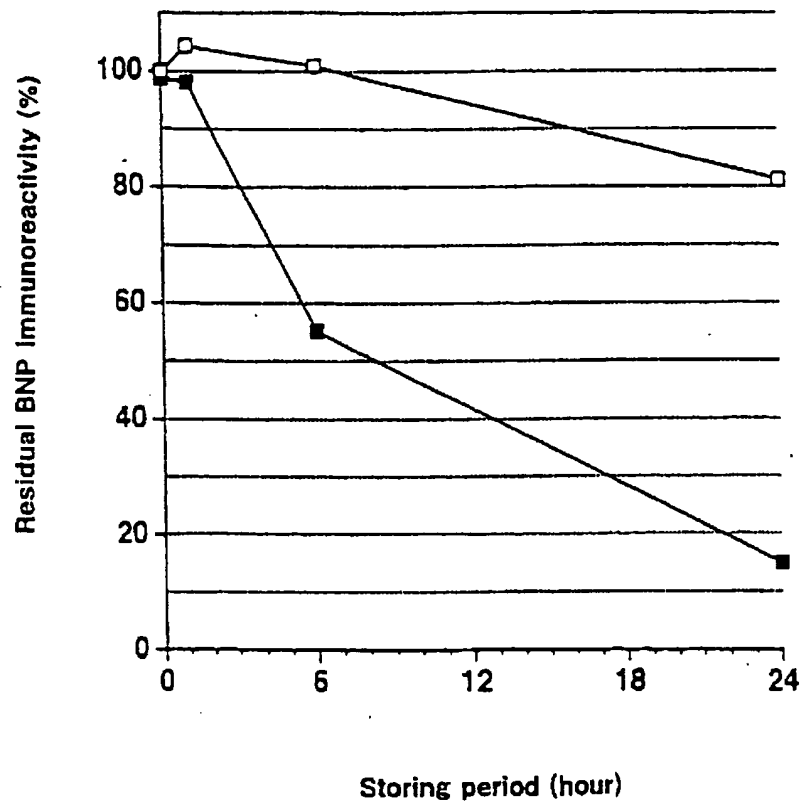


Figure 2

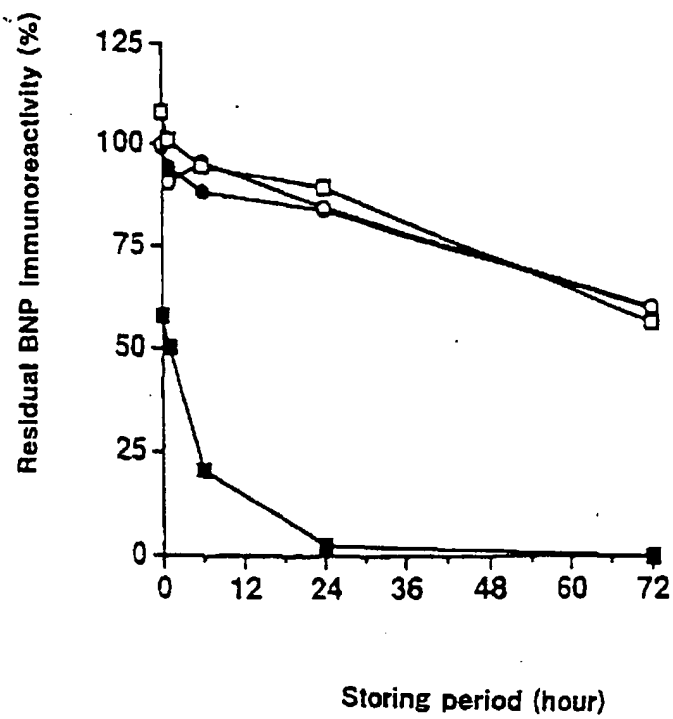
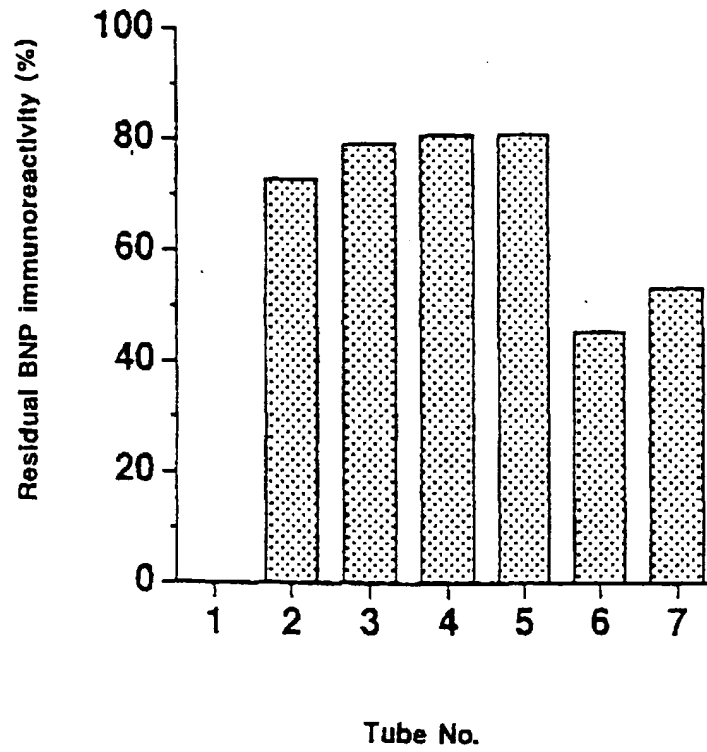


Figure 3





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP98/01470

## A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.<sup>1</sup> G01N33/48, 33/68, 33/53

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.<sup>1</sup> G01N33/48, 33/68, 33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1922-1996 Toroku Jitsuyo Shinan Koho 1994-1998

Kokai Jitsuyo Shinan Koho 1971-1998 Jitsuyo Shinan Toroku Koho 1996-1998

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSYS PREVIEWS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Clin. Chem., Vol. 42, No. 10 (1996) p.1627-1633	1, 3, 5-8
Y		4
A		2
Y	Biochemical and Biophysical Research Communications, Vol. 161, No. 3 (1989) p.1177-1183	4
A	Pharmacology & Toxicology Vol. 68, No. 4 (1991) p.276-281	1-8

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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"Z" document member of the same patent family

Date of the actual completion of the international search  
June 9, 1998 (09. 06. 98)Date of mailing of the international search report  
June 23, 1998 (23. 06. 98)Name and mailing address of the ISA/  
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